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(71) Applicant: OLIGOS ETC. AND OLIGOS THERAPEUTICS, INC. [US/US]; 29970 S.W. Town Center Loop W., Wilsonville, OR 97070 (US).

(72) Inventors: ARROW, Amy; 15 Equestrian Ridge Road, Newton, CT 06470 (US). DALE, Roderic, M., K.; 26761 S.W. 45th Drive, Wilsonville, OR 97070 (US). THOMPSON, Theresa, L.; 2222 S.W. Ek Road, West Linn, OR 97068 (US).

(74) Agents: FRIEBEL, Thomas, E. et al.; Pennie & Edmonds LLP, 1155 Avenue of the Americas, New York, NY 10036 (US).

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(54) Title: ANTISENSE OLIGONUCLEOTIDES AS ANTIBACTERIAL AGENTS

(57) Abstract

A novel method is provided that teaches the therapeutic use of nuclease resistant oligonucleotides for treating animals having an infection caused by a pathogenic bacterium. The method involves the integration of (1) methods for selecting the correct oligonucleotide, (2) synthesis and purification of nuclease resistant oligonucleotides, and (3) methods for *in vitro* analysis of potential antimicrobial oligonucleotides. The described oligonucleotides may comprise modified backbones, sugar residues, bases, or mixtures and have been subject to purification resulting in oligonucleotides that are capable of inhibiting the growth of a broad spectrum of clinically relevant bacterial species.

2.0. SUMMARY OF THE INVENTION

The present invention relates to methods for the treatment of animals, including humans, that have a bacterial disease. The preferred method of treatment comprises the administration of a purified antibacterial oligonucleotide having about 8 to about 80 nucleotides to the animal in an amount sufficient to inhibit bacterial growth, alleviate a symptom of the infection, or in an amount effective for treatment.

The purified antibacterial oligonucleotides of the present invention will preferably bear an enhanced ability to inhibit the growth of bacterial cells relative to previously disclosed oligonucleotide preparations. The present invention also represents the first disclosure of the use of oligonucleotides to inhibit the growth of intact clinica relevant bacteria. The oligonucleotides generally inhi bacterial growth by acting as antisense or antigene inhibitors of bacterial gene expression (when targete bacterial nucleic acid sequences), or by acting apta to alter the function of specific bacterial protein polypeptides (when associating target amino acid s contained in bacterial peptides, polypeptides, an Alternatively, the oligonucleotides are targeted to an antibiotic resistance gene to render the bacteria sensitive to a conventional antibiotic. In preferred embodiments, the antibacterial oligonucleotides are substantially nuclease resistant (*i.e.*, resistant to nuclease activity).

Additional embodiments of the present invention are antibacterial oligonucleotides that have been produced by a process that enhances the oligonucleotide's antibacterial activity. In particular, the presently described antibacterial oligonucleotides will be produced, or otherwise purified, by a process comprising either individually or in combination ion exchange or reverse phase chromatography, extractions, precipitations, gel filtrations, dialysis, diafiltration or functional equivalents. Column chromatography may be by traditional of methods or High-

whatsoever (other than growth in culture medium). In particular, a "wild-type" bacteria shall not be genetically modified such that the bacteria has an enhanced permeability to macromolecules or biological polymers or oligomers.

The term "antisense oligonucleotide" refers to an oligonucleotide that has a sequence that is substantially complementary to a target DNA or mRNA, so that the antisense oligonucleotide will hybridize in a complementary fashion to the DNA or mRNA to form a complex by Watson-Crick base pairing. Generally, the antisense oligonucleotide will bind the complementary target sequence with an avidity, *in vivo*, sufficient to inhibit the normal function of target sequence.

The term "bacteriostatic oligonucleotide" refers to oligonucleotides that inhibit or retard the growth of bacteria either *in vitro* or *in vivo*.

The term "bactericidal oligonucleotide" refers to oligonucleotides that directly, or indirectly, cause the death of bacteria either *in vitro* or *in vivo*.

The term "Gram negative bacteria" refers to the inability of bacteria to resist decolorization with alcohol after being treated with Gram's crystal violet stain. However, following decolorization, these bacteria can be readily counter-stained with safranin, imparting a pink or red color to the bacterium when viewed by light microscopy. This reaction is usually an indication that the bacterium's outer structure consists of a cytoplasmic membrane (inner), which is surrounded by a relatively thin peptidoglycan layer, which in turn, is surrounded by an outer membrane. Typical examples of Gram negative bacteria include, but are not limited to, *Escherichia, Salmonella, Edwardsiella, Arizona, Citrobacter, Enterobacter, Proteus, Yersinia, Klyvera, Klebsiella, Neiserria, Vibrio, Pasturella, Haemophilus, Pseudomonas, Moraxella, Eikenella, Fusobacterium, Acidominococcus, Actinobacillus, Cardiobacterium, Serratia, Providencia, Erwinia, Tatumella, Shigella, Branhamella, Aeromonas, Francisella, Gardnerella, Alcalígenes, Kingella, Agrobacterium, Leptotrichia, Megasphaera, Capnocytophaga,*

Cromobacterium, *Hafnia*, *Morganella*, *Pectobacterium*, *Cadecea*, *Helicobacter*, *Morococcus*, *Pleisiomonas*, *Bordetella*, *Brucella*, *Achromobacter*, *Flavobacterium*, *Bacteroides*, *Veillonella*, *Streptobacillus*, *Pneumococcus*, and *Calymmatobacterium*.

The term "Gram positive bacteria" refers to the ability of bacteria to resist decolorization with alcohol after treatment with Gram's crystal violet stain, imparting a violet color to the bacterium when viewed by light microscopy. This reaction is usually an indication that the bacterium's outer structure consists of a cytoplasmic membrane surrounded by a thick, rigid bacterial cell wall mainly comprised of peptidoglycan (murein). Typical examples of Gram positive bacteria include, but are not limited to, *Aerococcus*, *Listeria*, *Streptomyces*, *Actinomadura*, *Lactobacillus*, *Eubacterium*, *Arachnia*, *Mycobacterium*, *Peptostreptococcus*, *Staphylococcus*, *Corynebacterium*, *Erysipelothrix*, *Dermatophilus*, *Rhodococcus*, *Bifodobacterium*, *Lactobacillus*, *Streptococcus*, *Bacillus*, *Peptococcus*, *Micrococcus*, *Kurthia*, *Nocardia*, *Nocardiopsis*, *Rothia*, *Propionibacterium*, *Actinomyces*, *Enterococcus*, and *Clostridia*.

Additionally, the presently described antibacterial oligonucleotides may be effective against bacteria including, but not limited to, *Campylobacter*, *Spirillium*, *Borrelia*, *Treponema*, *Leptospira*, *Legionella*, and *Chlamydia*.

The term "mycobacterium" refers to any and all strains of bacteria drawn from the group comprising: *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium leprae*, *Mycobacterium avium-intracellulare*, *Mycobacterium kansasii*, *Mycobacterium scrofulsceum*, *Mycobacterium marinum*, *Mycobacterium fortuitum*, *Mycobacterium ulcerans*, *Mycobacterium chelonae*, *Mycobacterium paratuberculosis*, *Mycobacterium xenopi*, *Mycobacterium simiae*, or other mycobacteria falling within the Runyon groups I-IV as described in Runyon, Med. Clin. North Amer. 43:273-290 (1959), or Mandell *et al.*, 1990, Principles and Practice of Infectious Disease 3rd. ed., Churchill Livingstone Inc., New York, N.Y. 10036, herein incorporated by reference.

Oligonucleotides by High-Performance Liquid Chromatography", *In* Methods in Molecular Biology, vol. 26: Protocols for Oligonucleotide Conjugates, S. Agrawal ed., Humana Press, Inc., Totowa, NJ; Aharon *et al.*, 1993, J. Chrom. 698:293-301; and Millipore Technical Bulletin, 1992, "Antisense DNA: Synthesis, Purification, and Analysis"). Peak fractions were combined and the samples were desalted and concentrated by alcohol (ethanol, butanol, isopropanol, and isomers and mixtures thereof, etc.) precipitation, diafiltration, or gel filtration followed by lyophilization, or solvent evaporation under vacuum in commercially available instrumentation such as, for example, a Savant Speed Vac.

Oligonucleotides of the invention were dissolved in pyrogen free, sterile, physiological saline (*i.e.*, 0.85% saline) and sterile filtered through 0.2 micron pyrogen free filters.

4.4. Oligonucleotides As Antibiotics

The principal criteria for designing antisense oligonucleotides for treating bacterial infections are: (1) retention of sequence-specific base-pairing and triplex-forming interactions; (2) increasing nuclease stability; (3) increasing the extent or kinetics of entry into the target cell; (4) activating RNase H (while a consideration, a given oligonucleotide's ability to activate RNase H is not strictly required to observe antibacterial activity); and (5) ease of synthesis and purification.

Although exquisite sequence specificity may be preferred in some instances, the presently described oligonucleotides are capable of specifically inhibiting bacterial growth as long as they remain capable of associating with the target sequence under the relevant conditions. For example, the use of oligonucleotides to degrade RNA simply requires that the oligonucleotide associate (with at least a four base match) with the bacterial RNA long enough to activate RNase H. Thus, oligonucleotides that harbor relaxed sequence specificity are deemed sufficient to activate RNase H. In

fact, because not all bacterial target sequences are known, applications are contemplated where the antibacterial oligonucleotide provides the desired inhibitory effect although not specifically targeted, or homologous, to a given bacterial gene.

Modified oligonucleotides that activate RNAse H are advantageous because such oligonucleotides will hybridize to their target mRNAs and create a substrate that can be digested by RNase H. RNase H digestion destroys the target mRNA, and thus, these oligonucleotides prevent the translation of the target mRNA. Accordingly, protein expression is inhibited either by the enzymatic destruction of the target mRNA, or by the oligonucleotide physically blocking translation (*i.e.*, after the oligonucleotide directly associates with ribosomal sequence).

Although RNase H activation is a factor in the design of antibacterial oligonucleotides, many antibacterial oligonucleotides (e.g., ribonucleotides targeting bacterial RNA) are not designed to activate RNase H. Typically, modified oligonucleotides that are connected by stretches of unmodified phosphodiester linkages comprising at least about four nucleotides to about seven nucleotides should retain the ability to activate RNase H. Also, it has been observed that phosphorothioate ribonucleotides can also activate RNAase H digestion. The differential specificity of mammalian RNase H (minimum of 5 bases) and bacterial RNAase (4 bases) affords a means of selectively targeting bacterial genes that may have strong sequence homology with certain animal genes.

Also contemplated are modified oligonucleotides that can form triplexes with duplex DNA (antigene oligonucleotides), and oligonucleotides that can be used as ribozymes.

Another embodiment of the presently described antibacterial oligonucleotides is aptameric oligomers that are capable of effectively mimicking protein domains and exerting an antibacterial effect by directly associating with bacterial proteins or structures.

Additionally, antibacterial oligonucleotides may exert a therapeutic effect by specifically binding and deactivating cellular machinery. For example, the presently described oligonucleotides may directly bind ribosomal sequences and inhibit translation by stearically hindering translation initiation, elongation, disassociation, or by directly destabilizing the structure of the bacterial ribosomes.

Antibiotic resistance is often caused by the presence of resistance factors that render an antibiotic ineffective. By targeting resistance factors, the presently described oligonucleotides may render an otherwise antibiotic resistant organism sensitive to conventional antibiotics. Accordingly, another embodiment of the present invention is the use of antibacterial oligonucleotides in conjunction with conventional antibiotics.

Another embodiment of the present invention involves the use of the presently described oligonucleotides to inhibit the expression of genes whose products regulate the replication or transfer of bacterial genes. Additionally, given that antibiotic resistance genes or other virulence factors are often encoded by plasmids, antibacterial oligonucleotides targeted against plasmid replication, transfer (by conjugative transfer), or gene expression are particularly of interest. Similarly, antibacterial oligonucleotides are contemplated that are capable of inhibiting the expression and transfer of genes encoded by transposable genetic elements (*e.g.*, transposons).

4.4.1. Selection Of Targets For Oligonucleotides: Gene/Operon Target Identification

Antisense oligonucleotides which target essential structural genes, metabolic pathway genes, or transport system genes will inhibit the growth of bacterial cells. For pathogenic bacteria, virulence factors such as, for example, genes encoding antibiotic resistance, toxins, adherence and invasion factors, pili or fimbriae, flagella, antigenic variation factors, and iron binding factors, are also

preferred targets. These targets should be pathogen specific, and thus oligonucleotides directed against these targets will preferably not harm either host cells, or the normal bacterial flora of the gut.

While some bacterial genes are expressed as individual transcripts, many are transcribed as part of a multicistronic unit or operon. Examples include the ribosomal protein operons, such as the *str* operon and the alpha operon in *Escherichia coli*. Where possible operon transcripts are targeted. Disruption of expression of a gene in the operon may also adversely effect the expression of other genes encoded within the same operon (often in operon transcripts the translation of the 5'-most genes are required for efficient translation of the downstream genes). In theory this could result in pleiotropic growth effects from a single oligonucleotide sequence. Specific genes and transcripts (whether expressed as part of an operon or independently) are targeted on the basis of their function in the cell. For example, the gene for glucose-6-phosphate dehydrogenase is central to sugar metabolism. Other genes may not be relevant in our normal assay system; disruption of lactose metabolism is expected to have only a minor effect, if any, on *Escherichia coli* growth in media containing a more readily available carbon source such as glucose.

Once a target gene or operon has been selected, a target region within the gene or operon sequence must be selected, for example, the start codon. An analysis of the sequences around the target sequence (*e.g.*, 5' untranslated region, start codon, internal sequence feature, termination codon, 3' untranslated region) is performed. This analysis generally encompasses a total of about 120 bases that flank the target sequence. This analysis further predicts the secondary structure of the antisense oligonucleotide, and can be performed using commercially available computer software. The extended target sequence is checked for regions of stable secondary structure. The positions of the bases predicted to be involved in the stem-and-loop structures should be marked

and the predicted Tm of the structures noted. Preferably, stem sequences should be avoided where possible. Moreover, predicted secondary structures with predicted melting temperature of 45°C or less are disregarded in this analysis.

A maximum oligonucleotide length is also selected, and the program identifies the clear regions (no stems, or the structures with the lowest melting temperatures), and also checks the loop melting temperatures for the generated oligonucleotides. Such programs are well known in the art and include, for example, the program *OligoTech* version 1.0 (Copyright© 1995, Oligos Etc. Inc. & Oligo Therapeutics Inc.).

The length of the flanking sequence to be analyzed may be increased if an oligonucleotide with a length of greater than 30 bases is selected. The transcription start site and termination site (or any attenuation sequence) are generally the most distal sequences that will be analyzed. On occasion, this may result in an analysis of about 190 or more bases of flanking sequence.

Potential oligonucleotide sequences that have high loop melting temperatures may be eliminated by the above analysis. Note that the melting temperatures for the loops obtained for the commercial programs may need to be adjusted for modified oligonucleotides since these oligonucleotides may have altered base pairing avidities.

Several additional characteristics of the oligonucleotides are also considered. Stable secondary structure (potentially stable under physiologic conditions), runs of a single base (*e.g.*, 4 or more A's), and sequences that potentially form stable homodimers are also eliminated if possible. (In cases where double-strand oligonucleotide is the desired end result, homodimers may be preferred.) The base composition of the oligonucleotide is also checked.

The two or three oligonucleotide sequences that most nearly meet the above criteria are selected. Using these final oligonucleotides, the program analyzes each sequence and notes loop melting temperatures for both the sense and

the antisense strands of the candidate sequences. This decreases the possibility of the computer analysis missing a potential problem structure.

The candidate sequences, selected as above, are searched for sequence matches in available sequence databases (for example, Genbank) using commercially available search software. The first search is against the bacterial sequence database(s). This allows the identification of other targets that may also be affected by the candidate sequence, and may also indicate which sequences are potentially effective across bacterial genera. Since many different bacterial genera have highly related genetic organizations or related gene sequences, a potential oligonucleotide may be effective against multiple bacterial genera. For example, the sequences of the *gyrA* genes of *Escherichia coli* and *Salmonella typhimurium* are essentially identical near the start codon.

Additionally, since bacterial translation occurs simultaneously with transcription, it may be generally preferable to target antisense oligonucleotides to bacterial sequences at or near the Shine-Delgarno site (ribosome binding site) or to the translation start site of the targeted transcript.

The second search is versus a database including human/primate sequences. Since these databases are still quite limited (relative to the entire amount of sequence data in the genome), databases generally including mammalian sequences should be searched. Oligonucleotides that have high specificity matches to relevant mammalian sequences should be eliminated from initial consideration. (Note: that they may be re-included after further evaluation of the possible target sequences.)

As a consequence of the incomplete nature of the data bases comprising bacterial, primate, rodent, and mammalian sequences, this method cannot ensure that all potential targets or conflicts are identified. However, as sequence data accumulates, this method will allow an experienced

What is claimed is:

1. A method for treating an animal, including a human, having an infection caused by a pathogenic bacterium, comprising: administering to the animal a composition comprising a pharmaceutically acceptable carrier and a substantially nuclease resistant oligonucleotide having about 8 to about 80 nucleotides and targeted to a nucleic acid or protein in the bacterium in an amount sufficient to alleviate a symptom of the infection.

2. The method of claim 1, wherein the nucleic acid or protein is involved in the synthesis, metabolism, assembly or regulation of at least one of the group consisting of energy, DNA replication, cell division, regulatory proteins, cell walls, sugars, virulence, fatty acids, mRNAs, tRNAs, rRNAs, ribosomal proteins, proteins involved in protein synthesis, phospholipids, periplasmic proteins, secretory proteins, flagellar proteins, transport proteins, amino acids, lipopolysaccharides, purines, pyrimidines, pili, outer membrane proteins, nitrogen, antibiotic binding proteins and vitamins.

3. The method of claim 1, wherein the oligonucleotide is capable of associating with a nucleic acid or protein in the bacterium such that it inhibits at least one of the group consisting of bacterial growth, reproduction, metabolism, synthesis of toxins, progress of infection and virulence.

4. The method of claim 3, wherein the associating is hybridizing to an mRNA in the bacterium at or near the initiation codon, in the 5' untranslated region, in the 3' untranslated region, internal to the coding region or an intermediate region of the mRNA.

5. The method of claim 3, wherein the associating is hybridizing to DNA in the bacterium.

6. The method of claim 5, wherein the hybridizing forms a triplex structure.

7. The method of claim 3, wherein the associating is binding with a protein in the bacterium.